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Fetal Wound Healing

The Ontogeny of Scar Formation in the Non-Human Primate

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Objective

This study determined how scar formation develops in a non-human primate model of fetal skin repair.

Summary Background Data

A transition from healing scarlessly to healing with scar formation characterizes skin repair in rat and sheep fetuses. New knowledge of the regulatory processes occurring in the fetal wound at the initial stages of scar formation may provide insights into the early mechanisms of scar formation.

Methods

Full-thickness wounds were made in fetal rhesus monkey lips from 75 through 114 days gestation (n = 6, term = 165 days). Wounds were harvested at 14 days postwounding and processed for histology (hematoxylin & eosin, Masson's trichrome) as well as immunohistochemistry (human type I or type III collagen).

Results

Wounds healed with complete restoration of normal tissue architecture in the 75-day gestation fetus. However in the 85–100-day gestation fetuses, wounds healed with an absence of hair follicles and sebaceous glands, but the dermal collagen pattern remained reticular and similar to that in unwounded dermis. At 107 days, a thin scar was present in the wound, thereby demonstrating a transition to scar formation between 100 and 107 days gestation (early 3rd trimester) in the non-human primate.

Conclusions

In the non-human primate fetus, a transition from scarless repair to adult-type repair with scar formation occurs in the early third trimester. These data provide insight into the transition process; the ontogeny of scar formation is characterized initially by wounds healing without the presence of epidermal appendages but with a normal reticular dermal collagen pattern, which we term the "transition wound."

Fetal wound repair is noteworthy for an absence of scarring and fibrosis. Since the human fetus is a surgical patient,[1–3] the specter of *in utero* repair of human facial defects to take advantage of these unique healing characteristics of the fetus has been raised by several investigators.[4–7] However, before *in utero* repair of human fetal cleft lip and palate can be attempted, several questions about the biology of fetal repair must first be addressed,

not withstanding demonstration of appropriate maternal and fetal safety during intervention for a non–life-threatening malformation. Questions that must be answered are: Does the the primate fetus heal skin wounds without scar? And if so, when during gestation does scarless repair occur?

A transition from scarless repair early in gestation to healing with scar formation later in gestation has been documented in two animal models of fetal wound repair. In the rat fetus (term = 20 days), the transition occurs on day 18–19,[8] which is difficult to extrapolate to the human situation. In the long gestation sheep fetus (term = 145 days), the transition occurs between 100 and 120 days gestation for incisional wounds, which corresponds to the mid-third trimester.[9] We have documented scarless repair in human fetal skin up to 22 weeks gestation in experiments in which the human fetal skin is transplanted onto athymic mice and subsequently wounded.[10] However, we have not demonstrated a transition to scar formation in this *ex utero* system. Before performing human fetal surgery to take advantage of scarless repair, the transition period in primate fetal repair must be documented. Moreover, characterization of the crucial cellular and matrix events during this transition period may provide insights to help modulate adult wound repair to become more fetal-like.[11]

In the present study, we develop a model of fetal wound healing using the rhesus monkey to most closely approximate the developing human condition. Scar is characterized by densely packed, disorganized collagen bundles in the wound with an absence of hair follicles, sebaceous glands, and other appendages. We document scarless lip repair in the mid-second trimester at which time there is full morphologic regeneration of normal tissue architecture. We find a gradual transition to adult-type scar formation by the beginning of the third trimester and demonstrate a novel repair outcome that occurs during the repair process, which we propose to call the *transition wound.*

MATERIALS AND METHODS

Animals

Six time-dated pregnant rhesus (*Macaca mulatta*) monkeys were used in this study. Animals were housed at the California Primate Research Center (Davis, CA), where they were fed food and water *ad libitum* with daily postoperative veterinary evaluations. The animals were fasted for 12 hours before surgery.

Fetal Wounds

Wounds were made in fetuses at 75, 85, 90, 100, 107, and 114 days gestation (term = 165 days). Lip wounds were chosen for ease of replication and unique structure with epithelium on both surfaces and skeletal muscle in the center. The same fetal surgical, anesthetic, and tocolytic techniques that we developed in non-human primates as a prelude to human fetal surgery were used in this study.[12] Briefly, the pregnant monkey was immobilized with ketamine (10 mg/kg) administered intramuscularly. Peripheral intravenous access was obtained percutaneously and 250 cc of lactated Ringer's solution was administered during the procedure. Indomethacin (5 mg) was given intravenously pre- and postoperatively for tocolysis. Under isoflurane endotracheal anesthesia, the maternal abdomen was sterilely prepped and draped, and a maternal laparotomy was made to expose the gravid uterus. The site and orientation of the hysterotomy was determined by preoperative sonographic localization of the two placental discs with care to place the hysterotomy away from these structures. After hysterotomy, amniotic fluid was withdrawn, kept warm, and returned during uterine closure. The fetal head and neck was exteriorized for wounding with careful attention to preserve umbilical blood flow with as little fetal manipulation as possible. The exposed fetus and uterus were kept warm with constant warm saline irrigation.

Under x3.5 loupe magnification, a single full-thickness upper-lip wound was made on each fetus; a 2-mm wide paramedian section, including the vermilion border and the nasal sill, was excised. The wound edges were reapproximated with interrupted 7-0 Surgilene® sutures (Davis-Geck, Manati, PR).

The hysterotomy was closed in two layers with 3-0 Maxon® (Ethicon, Sommerville, NJ) taking great care to seal the amniotic membranes after the amniotic fluid volume was replaced. The maternal laparotomy fascia layer was closed with running 2-0 Vicryl® (Ethicon, Sommerville, NJ), and the skin was closed with a 3-0 Vicryl® subcuticular suture.

Adult Wounds

Control adult incisional wounds were made on the maternal thorax and closed with interrupted 3-0 Vicryl® sutures.

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Harvest

Fetal lip wounds were harvested at 14 days post-wounding. Each animal represents a separate time point. At the time of harvest, the maternal monkey underwent general endotracheal anesthesia as described earlier. After laparotomy and hysterotomy, fetectomy was performed. The maternal hysterotomy and laparotomy incisions were closed and the adult monkey returned to her breeding colony.

The fetal and adult wounds were bisected immediately and the halves fixed in either 10% buffered formalin or snapfrozen in isopentane which had been precooled in liquid nitrogen. Frozen wounds were stored at −80 C until analysis.

Histology

Formalin-fixed sections were embedded in paraffin, and 7 μm sections were stained with either hematoxylin and eosin or Masson's trichrome (which stains connective tissue and collagen).

Immunohistochemistry

Indirect immunohistochemical technique was used to specifically stain for either collagen type I or type III in the wounds. Briefly, frozen tissue was embedded in OCT compound (Miles Inc., Elkhart, IN) and 7 μm sections were cut and acetone-fixed. To block nonspecific background staining, goat non-immune serum was applied for 30 minutes before applying primary antibody. Primary antibodies were polyclonal, raised in rabbits against human collagen type I and against human collagen type III (Chemicon, Temecula, CA). The specificity for each antibody has been previously characterized.[13,14] Primary antibodies were applied for 60 minutes in a humidified chamber. Sections were washed three times (5 minutes each) with phosphate-buffered saline (PBS), and then secondary antibody (goat anti-rabbit conjugated fluorescein isothiocyanate antibody-Sigma, St. Louis, MO) was applied for 60 minutes. Sections were washed and mounted in a nonfading medium (1,4-diazobicyclo-[2,2,2]-octane). Wounds were photographed under identical conditions on a Zeiss photomicroscope II using Kodak Kodachrome® ASA 800 film (Eastman Kodak, Rochester, NY).

Negative controls consisted of substituting nonimmune rabbit serum (Cedar Lane, Westbury, NY) (irrelevant antibody) instead of primary antibody. These control sections did not show significant staining. Internal positive controls were present on each section as both collagen type I and type III are widely distributed in normal, unwounded skin.[15] Each section of wound and adjacent normal skin displayed this pattern.

RESULTS

All wounds were completely healed with restoration of epidermal and dermal continuity at 14 days. The repaired fetal lip wounds were grossly indistinguishable from the contralateral lip segment. An epidermal furrow marked the wound sites. There were no differences between the respective type I and type III collagen patterns at each harvest time point.

Regeneration

The 75-day gestation wound healed with reformation of normal dermal and epidermal architecture. The patterns of hair follicles and sebaceous glands present in the wounds were unchanged from the surrounding dermis (Fig. 1A). The collagen deposition pattern was also identical to unwounded dermis with its characteristic highly organized, reticular pattern (Fig. 2A). In addition, skeletal muscle present in the center of the lip healed without scar formation, further demonstrating scarless repair of mesenchymal components in the fetal repair process (Fig. 1A) other than connective tissue.

Transition

A change in the repair outcome with a shift from regeneration of all wound components to wounds healing with a loss of sebaceous gland and hair follicle patterns occurred in the 85- through 100-day gestation wounds. The absence of epidermal appendages was notable and allowed easy determination of wound location histologically (Fig. 1B). However, collagen immunohistochemistry demonstrated a normal, reticular collagen fiber deposition pattern in these wounds that was indistinguishable from unwounded dermis (Fig. 2B). These wounds displayed a "transition wound" in which the collagen deposition pattern was reticular (and thus scarless) but there was a conspicuous loss of appendages in the wound.

Scar

Classic scar formation occurred in the 107- and 114-day gestation wounds, signifying full transition to adult-type wound repair. These wounds not only healed without hair follicles and sebaceous glands (Fig. 1C), but also contained a thin band of disorganized, irregularly compacted collagen, which was distinct from the surround-

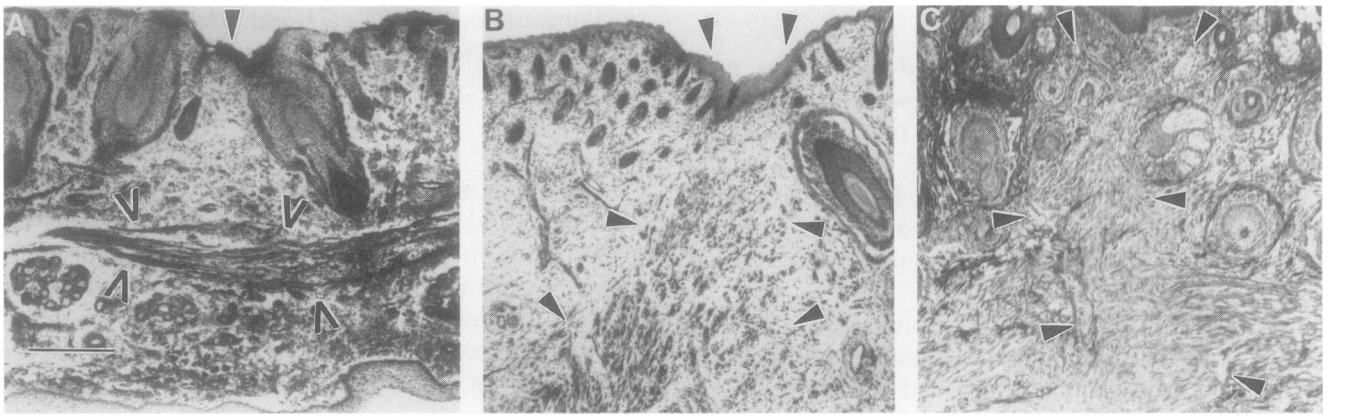

Figure 1. Lip wounds stained with Masson's trichrome to demonstrate connective tissue patterns. The cutaneous epidermis is superior and the oral mucosal surface is inferior in each photomicrograph. A: 75-day gestation wound (solid arrow). The hair follicle and sebaceous gland patterns are unchanged. In addition,striated lip muscle fibers have regenerated across the wound (open arrowheads), demonstrating regeneration of deeper mesenchymal components. B: 90-day gestation wound (arrows). The transition wound is demonstrated with its absence of appendages but retained ability to heal with a reticular collagen and connective tissue pattern. C: 114-day gestation wound (arrows). Appendages and circumoral muscle are separated by densely packed connective tissue scar. Bar, 100 μm.

ing dermis (Fig. 2C). All adult wounds healed with scar formation.

DISCUSSION

Although scarless fetal skin repair has been well described in several animal models,[16] the *in utero* transition from scarless repair to healing with scar formation has not been well characterized. By studying the temporal sequence of repair outcomes in lip wounds from mid-gestation through the mid-third trimester with histology and immunohistochemistry, we describe a novel wound repair outcome that has not been previously described in either the fetus or the adult: the transition wound. Early gestation fetal primate lip wounds can heal with regeneration of all dermal components including striated muscle, hair follicles, sebaceous glands, and collagen. However, as gestation proceeds, the primate fetus first loses its ability to regenerate normal hair follicle, sebaceous gland, and other appendage patterns but remains able to restore a normal, reticular collagen matrix pattern after wounding. This pattern of transition wound repair is neither regeneration nor is it classic scar since there is a reticular pattern of collagen immunostaining which is unchanged from unwounded dermis. By the early third trimester, a complete switch to adult-type repair has occurred. These fetal lip wounds heal with a loss of appendages and with densely packed, disorganized collagen deposition.

The transition process is gradual and spans several weeks in the primate fetus. During this period the cellular and matrix regulatory events that result in scar formation develop. The differences between fetal and adult dermal healing may reflect a more rapid and ordered deposition and turnover of tissue components, a less differentiated state of the wounded tissues, and an immature immune system. The role of hair follicles in scarless fetal dermal healing is unknown. Of interest, studies of wound healing in adult hair follicles have demonstrated scar-free healing of the damaged dermal papilla,[17] and the fibroblast-like cells of the papilla share characteristics associated with embryonic cell lines, such as cell aggregation in tissue culture.[18] Further studies focusing on the transition wound in this primate model should provide insights into the early mechanisms of scar formation.

Previous studies have correlated the absence of scarring in fetal wounds with the sparse inflammatory response, as evidenced by reduced macrophage and monocyte infiltrates,[19] absence of endogenous immunoglobulins at the wound site,[9,20] reduced angiogenesis, and altered levels of peptide growth factors.[21] Whitby and Ferguson studied the growth factor profile of fetal, neonatal, and adult mouse lip wounds by immunohistochemistry.[22] Platelet-derived growth factor was observed in all three groups. However, transforming growth factor type beta (TGF-β) and basic fibroblast growth factor (bFGF) were present in neonatal and adult wounds, but were not detected in fetal wounds. Exogenously applied TGF-β directly stimulates fibrosis in both fetal[23] and adult wounds,[24] and bFGF markedly enhances wound angiogenesis.[25] Fibrosis and angiogenesis are two features of adult repair that are not seen in scarless fetal

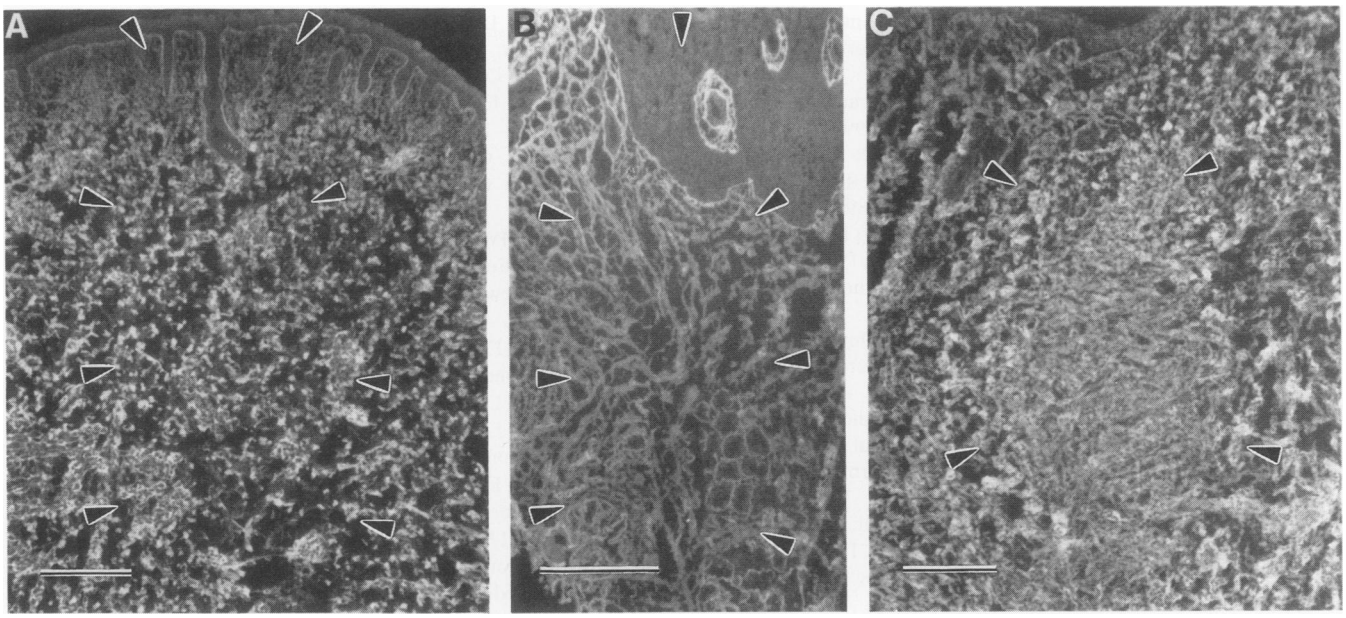

Figure 2. Lip wounds stained for collagen type I or III using immunohistochemistry. A: 75-day gestation wound (arrow), stained for collagen type III. The wound collagen pattern is reticular and unchanged from the surrounding dermis. B: 90-day gestation wound (arrows) stained for collagen type III. The collagen pattern in this wound remains reticular and unchanged from unwounded dermis. However, these wounds are noteworthy for a gap in appendages (Fig. 2B), showing the inability of the fetus to completely regenerate all dermal components at this time point. C: 114-day gestation wound (arrows) stained for collagen type I. Densely packed, disorganized collagen fibers are present in the wound as a thin scar. These fetal wounds have completed the transition to an adult-type wound healing outcome. Bars, A & C: 100 μm; B: 50 μm.

repair. Interestingly, experimental reduction of TGF-β concentration within healing adult rat wounds by application of neutralizing antibody to this cytokine results in markedly reduced scarring.[26] By further analyzing and comparing the inflammatory response and changing cytokine profiles between scarless wounds, transition wounds, and scar wounds in our non-human primate model, insights into the early regulatory mechanisms that lead to scar formation may be obtained. More sophisticated manipulations of postnatal wound repair speed and outcome by growth factor augmentation or ablation can then be studied.

The prospect of *in utero* repair of fetal cleft lip and palate has been raised.[4–7] To date, human fetal surgery has only been performed in highly selected patients for life-threatening fetal conditions (38 cases; 18–28 weeks gestation).[1–3] Before fetal surgery for nonlethal conditions can be performed, maternal-fetal safety must be ensured at acceptably low levels,[27] which may be possible with a new endoscopic surgical approach to the fetus.[28]

In addition to maternal/fetal safety concerns, the impact of the proposed intervention on the natural history of the lesion being treated must be examined. This study demonstrates for the first time that there is a sequential loss of regenerative capabilities in non-human primate fetal skin. These findings have implications for the timing of possible repair of human craniofacial anomalies. Extrapolation to the human fetus means that *in utero* repair performed during the third trimester would be associated with classic scar formation and contracture, thus negating the benefits of scarless repair and imposing the added maternal/fetal risks of *in utero* intervention.

As human fetal surgery has become more established, the study of fetal wound repair has become clinically relevant and generated interest in both surgical and basic science laboratories. The prospect of scarless wound repair is exciting because scar and fibrosis are the end result of tissue injury and destruction in virtually all organ systems.[11] An understanding of both the fetal wound healing "blueprint" of ideal tissue repair and the ontogeny of scar formation may lead to therapeutic strategies to help avert scarring and fibrosis in adult wounds.

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